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Spectroscopic studies on the interaction of U(VI) with *Bacillus sphaericus*

By P. J. Panak[1,*], R. Knopp[1], C. H. Booth[1] and H. Nitsche[1,2]

[1] Lawrence Berkeley National Laboratory, The Glenn T. Seaborg Center, Berkeley, CA 94720, USA
[2] University of California at Berkeley, Department of Chemistry, Berkeley, CA 94720, USA

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Summary. We studied the interaction of U(VI) with vegetative cells, heat killed cells, spores, and decomposed cells of *Bacillus sphaericus*. The characterization of the formed complexes was performed by time-resolved laser fluorescence spectroscopy (TRLFS) and extended X-ray absorption fine structure spectroscopy (EXAFS). We observed no significant differences in the sorption behavior of vegetative and heat killed cells, whereas the spores showed a higher sorption of U(VI) (related to their dry weight). Regardless of the higher relative sorption of the spores of *B. sphaericus*, the fluorescence and EXAFS spectra of the vegetative cells, heat killed cells and spores were almost identical. Analysis of the data proved that U(VI) forms inner sphere complexes with organic bound phosphate groups on the cell surface. We observed no significant differences in the coordination numbers and the distances of the oxygen and phosphorus atoms in the inner coordination sphere.

After eight weeks, the vegetative cells of *B. sphaericus* were completely decomposed. Lysing of the cell walls and activity of enzymes led to a release of various decomposition products. We found that large amounts of $H_2PO_4^-$ were released which caused a quantitative precipitation of bacterial U(VI) as $UO_2(H_2PO_4)_2$. The $H_2PO_4^-$ was detected by Raman spectroscopy. The decomposed bacterial suspension showed the same fluorescence spectrum as $UO_2(H_2PO_4)_2$ which differed significantly from those of the bacterial U(VI) surface complexes.

Introduction

Microbial activity in soil, sediment and water can have an appreciable effect on the dissolution and precipitation of actinides [1]. Both processes can be caused by various mechanisms, such as oxidation and reduction of metals, changes in pH and E_h (both affecting the solubility), interaction with microbial metabolites, biopolymers, or decomposition products, biosorption and bioaccumulation and formation of stable minerals [2]. Detailed information on the mechanism of biotransformation is necessary for predicting the microbial impact on the migration behavior of actinides in the environment as well as developing remediation and decontamination strategies for contaminated sites. The key processes leading to the immobilization of actinides such as bioaccumulation, biosorption, precipitation and mineral formation are of particular interest. Our recent studies on the interaction of Pu(VI) and U(VI) with *B. sphaericus* have shown that phosphate complexes are formed [3–5]. The cell walls of bacteria are porous three-dimensional macromolecular networks carrying carboxyl, phosphate and amino groups. We determined the phosphate content of the cell wall of the *B. sphaericus* strain to be $0.89 \pm 0.09\,\mathrm{mmol/g_{dry\ weight}}$ [5]. On the other hand, phosphate is an essential nutrient in the metabolism of bacteria. Excretion of phosphate can cause a precipitation of actinide phosphates on the surface of the bacterial cells which was already considered for remediation purposes to precipitate actinides [6].

Furthermore, the complexes formed also depend on the age and the status of the bacterial strain. *Bacilli* form spores under harsh conditions which might influence the interaction with actinides. Besides living cells and spores, also dead and decomposed cells are present in natural systems. Thereby, the interactions with all kinds of cells of *B. sphaericus* and decomposition products have to be considered for predicting a possible impact of bacteria on the transport of actinides.

Our research focuses on the interaction of *B. sphaericus* with U(VI) as a function of age and status of the cells. Sorption studies with U(VI) provide information on the amount of uranium taken up by living cells, spores, dead and decomposed cells. We used time-resolved laser fluorescence spectroscopy (TRLFS) and X-ray absorption spectroscopy to characterize the complexes formed. EXAFS (Extended X-ray Absorption Fine Structure Spectroscopy) provides information on the chemical environment of U(VI), in particular bond lengths and the number of neighboring atoms. In addition, with TRLFS we can differentiate between complexes with organo-phosphate groups on the cell surface and the formation of uranyl phosphate precipitate. Combining both methods, detailed knowledge of the different processes resulting from the interaction of U(VI) with *B. sphaericus* in natural systems is obtained.

*Author for correspondence (E-mail: panak@ine.fzk.de).
Current address: Forschungszentrum Karlsruhe, Institut für Nukleare Entsorgung, P.O. Box 3640, D-76021 Karlsruhe, Germany

Experimental

The *Bacillus sphaericus* strain (*Bacillus sphaericus* ATCC 14577) was grown under aerobic conditions in 500 mL nutrient medium (8 g/L nutrient broth, Difco) at 22 °C. To accelerate spore formation, the following components were added to the nutrient medium: $CaCl_2 \cdot H_2O$ (10^{-4} mol/L), $MgSO_4 \cdot 7H_2O$ (10^{-3} mol/L), $MnCl_2 \cdot 4H_2O$ (10^{-5} mol/L), Biotin (8.19×10^{-9} mol/L), Thiamine Hydrochloride (2.96×10^{-5} mol/L), $FeSO_4 \cdot 7H_2O$ (10^{-4} mol/L). The spore formation and the percentage of vegetative cells still left were observed by microscopy. After two weeks sporulation was completed and no vegetative cells could be detected. The biomass was harvested by centrifugation ($6000 \times$ g) and washed four times each with 50 mL physiological NaCl-solution (0.9%). Heat killed cells were obtained by heating the bacterial suspension at 60 °C for 48 hours. The intactness of the cells was confirmed by microscopy. The death of cells was proven by plating the bacterial solution on solid Nutrient Broth medium. No colonies were formed after the heating process.

To study biosorption, we used a U(VI) concentration of 0.14 mmol/L in 1.5 mL 0.9% NaCl-solution at pH 4.5. We varied the biomass from 0.03 $g_{dry\ weight}$/L to 1.56 $g_{dry\ weight}$/L (*Bacillus sphaericus*, vegetative cells), 0.05 $g_{dry\ weight}$/L to 1.56 $g_{dry\ weight}$/L (*Bacillus sphaericus*, spores), 0.03 $g_{dry\ weight}$/L to 1.56 $g_{dry\ weight}$/L (*Bacillus sphaericus*, heat killed cells) and 0.02 $g_{dry\ weight}$/L to 1.10 $g_{dry\ weight}$/L (*Bacillus sphaericus*, decomposed cells). The contact time of U(VI) with the biomass was 24 hours.

A pulsed Nd-YAG laser (Spectra Physics, GCR-3) was used for time-resolved laser fluorescence measurements. A wavelength of 355 nm (the third harmonic generation of the 1064 nm emission) was used for excitation. Emission spectra were recorded from 448.0 to 621.4 nm using a delay time of 2 μs and a gate width of 1 ms. Details of the TRLFS spectrometer are given elsewhere [7]. The samples contained 0.14 mmol/L of U(VI) and 0.78 $g_{dry\ weight}$/L (vegetative and heat killed cells, spores) and 0.80 $g_{dry\ weight}$/L (decomposed cells) of biomass. The pH was adjusted to 4.5. For a better detection of the bacterial uranyl complexes, we washed the biomass with 0.15 M $NaClO_4$ (pH 4.5) to remove the aqueous UO_2^{2+} in solution. In addition, two samples containing 0.14 mmol/L U(VI) each and 0.001 mol/L NaH_2PO_4 (leading to the formation of the $UO_2(H_2PO_4)_2$ precipitate) and 1.50×10^{-3} mol/L adenosine 5′-monophosphate were measured as references.

$UO_2(H_2PO_4)_2$ for X-ray absorption spectroscopy was precipitated at pH 4.5 by adding a 0.1 M NaH_2PO_4 solution to a 5×10^{-3} mol/L U(VI) stock solution. The bacterial samples were prepared by incubating 15 mL of the bacterial stock solutions (vegetative and heat killed cells: 2.34 $g_{dry\ weight}$/L; spores: 2.55 $g_{dry\ weight}$/L) in physiological NaCl solution with 3.93 mg U(VI) ([U] = 1.10×10^{-3} mol/L) at pH 4.5. After removing more than 90% of the water, the samples were measured as wet pastes. The uranyl adenosine 5′-monophosphate complex (U-AMP) was measured in solution containing 2.55×10^{-2} mol/L U(VI) and 2.40×10^{-3} mol/L AMP. Uranium X-ray absorption spectra were collected at the Stanford Synchrotron Radiation Laboratory on wiggler beam line 4-1 using a half-tuned Si (220) double-crystal monochromator. X-ray absorption measurements were performed in fluorescence mode at the U L_{III} edge. Energy calibration was performed by simultaneously measuring the XANES of a UO_2 reference. A four-pixel Ge fluorescence detector [8] was used for data collection. Three scans were collected on each sample and averaged to improve the statistics. All data reduction and fits in this paper utilize standard procedures [9, 10]. In particular, data from the Ge detector was corrected for dead time. Curve-fitting amplitudes and phases were calculated using FEFF 7 [11].

Results

Sorption studies

The sorption of uranium(VI) by vegetative cells, heat killed cells, spores, and decomposed cells of *B. sphaericus* as a function of biomass at pH 4.5 is presented in Fig. 1. For a better comparison, the results are normalized to the dry weight of the biomass. Microscopic studies have shown that the biomass agglomerates with increasing biomass concentrations. Due to the strong agglomeration, the relative biosorption efficiency decreases with increasing biomass concentrations. *B. sphaericus* forms very small circular spores. In particular at low biomass concentrations, the spores of *B. sphaericus* show a higher biosorption compared to the vegetative cells. Comparing the sorption behavior of vegetative and heat killed cells, no significant differences are observed. These results confirm that uranium is bound by surface complexation with functional groups on

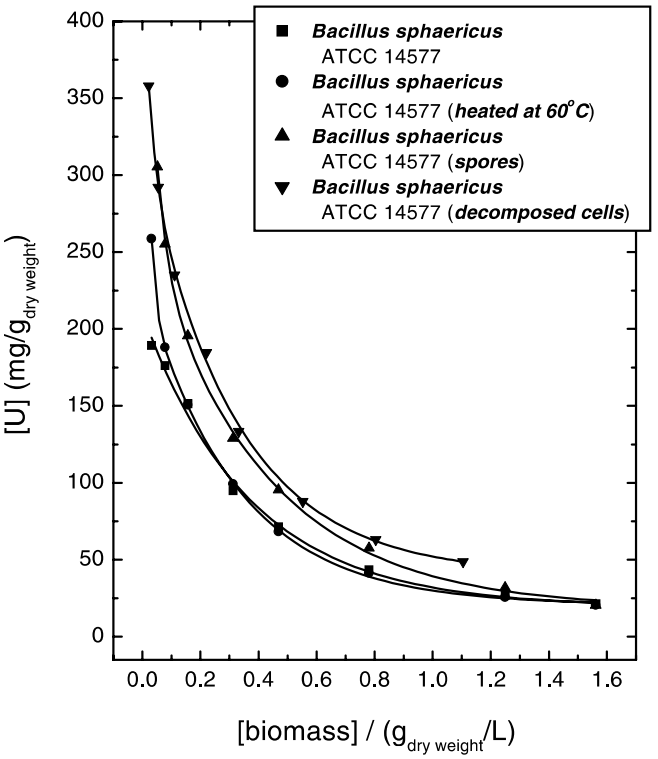

Fig. 1. Uranium(VI) biosorption of vegetative cells, heat killed cells, spores, and decomposed cells of *Bacillus sphaericus* ATCC 14577 at pH 4.5 as a function of the biomass concentration. The results are normalized to the dry weight of the bacteria.

the cell surface. No enzymatic action of the bacteria seems to be involved. After eight weeks, the decomposed cells of *B. sphaericus* remove a significant higher amount of U(VI) from the solution compared to the biosorption of the vegetative cells.

Time-resolved laser fluorescence spectroscopy

Whereas the sorption studies provide information on the amount of uranium taken up by the biomass under various conditions, a deeper insight into the coordination sphere of U(VI) complexes can be obtained by time-resolved laser fluorescence spectroscopy. A change in the chemical environment of U(VI) leads to a shift of the fluorescence peaks and to changes in the shape of the spectrum. Fig. 2 shows the fluorescence spectra of the bacterial U(VI)-complexes with vegetative cells, heat killed cells, spores, and decomposed cells of *Bacillus sphaericus* at pH 4.5 in comparison to the spectra of $UO_2(H_2PO_4)_2$ and Uranyl-AMP used as references. Interaction of uranyl with the vegetative cells of *Bacillus sphaericus* causes a strong bathochrome shift of the emission bands of 9 nm compared to the uranyl aquo ion (see Table 1). This bathochrome shift confirms the formation of stable inner-sphere complexes by replacement of water molecules of the inner coordination sphere by complexing ligands. In cell walls of Gram-positive bacteria (*e.g. B. sphaericus*) organic bound phosphate is a functional group that can lead to a strong complexation of U(VI). Teichoic acids (acidic polysaccharides) in the peptidoglycan layer of Gram-positive bacterial cell walls contain such organic bound phosphate groups [12]. Because adenosine monophosphate (AMP) has a phosphate group bound in a similar way, we use it as a model substance for teichoic acids. The shifts of the fluorescence peaks (Table 1) and the features of the spectra (Fig. 2) of U(VI) complexed by *B. sphaericus* and by AMP are almost identical. This means that both complexes display a comparable coordination with U(VI) and confirms the complex formation with organophosphate groups on the cell surface of the bacteria. In agreement with the sorption data, no significant differences are observed between the coordination of U(VI) with the vegetative and the heat killed cells. Microscopy of the heat killed cells show that the cell structure is not destroyed at a temperature of 60 °C. These results confirm that surface complexes are formed with vegetative cells and dead (but intact) cells with no metabolic activity involved. Though the biosorption (related to the biomass) of the small circular spores of *B. sphaericus* is higher than those of the vegetative and heat killed cells, the fluorescence spectra display a similar coordination with uranyl. This agrees with our former results on the interaction of U(VI) with vegetative cells and spores of different Bacillus strains [3]. Furthermore, the comparison of the spectroscopic parameters of the bacterial complexes with those of uranyl malonate (bidentate binding of carboxylic groups; see Table 1) confirms that the interaction of uranyl with *B. sphaericus* is determined by phosphate complexation.

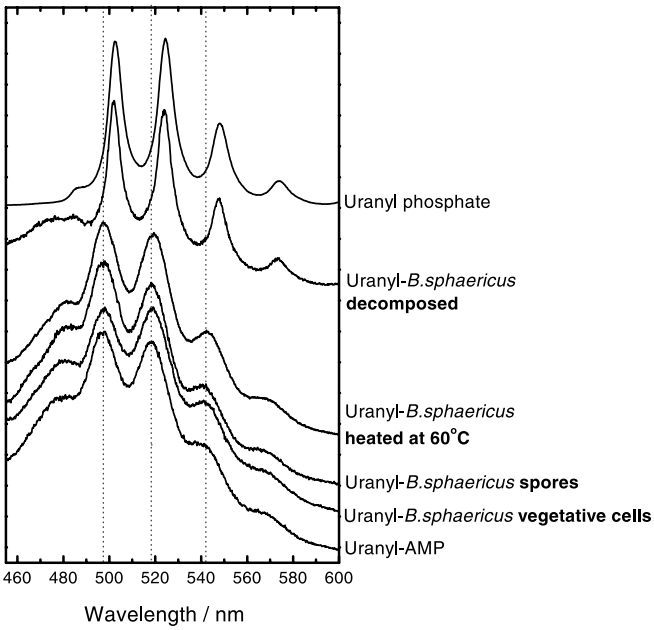

Fig. 2. Fluorescence spectra of U(VI) with vegetative cells, heat killed cells, spores, and decomposed cells of *Bacillus sphaericus* ATCC 14577 compared to the U-AMP and $UO_2(H_2PO_4)_2$ reference spectra.

The cells of *B. sphaericus* kept at room temperature without supplying nutrition die after a few days. The dead cells start lysing and excreting various decomposition products. Released enzymes (phosphatases) detach organic bound phosphates, *i.e.* from the DNA of bacteria, which are released as inorganic monomeric phosphate [13]. After 8 weeks all cells were completely fragmented. The corresponding spectrum of U(VI) differs significantly from those of the other U(VI) bacterial complexes. The emission peaks are shifted to higher wavelengths and have much smaller half widths (Table 1). The spectrum is consistent with the spectrum of the $UO_2(H_2PO_4)_2$ precipitate (Fig. 2, Table 1). These results confirm that during the bacterial decomposition process phosphate is excreted and uranyl phosphate

Table 1. Spectroscopic parameters of UO_2^{2+}, the bacterial UO_2^{2+}-complexes, the UO_2^{2+}-AMP complex, $UO_2(H_2PO_4)_2$, and UO_2^{2+}-malonate.

Sample	Emission maxima (nm) (Half width (nm))			
UO_2^{2+}	489 (11.4)	510 (11.5)	533 (15.6)	560 (14.1)
U-AMP	497 (10.7)	519 (11.4)	542[a]	569[a]
U-BS (vegetative cells)	498 (10.8)	519 (11.7)	542[a]	569[a]
U-BS (heat killed cells)	498 (10.9)	519 (11.8)	542[a]	569[a]
U-BS (spores)	498 (11.0)	519 (11.7)	542[a]	569[a]
U-BS (decomposed cells)	502 (5.9)	524 (7.1)	548[a]	574[a]
$UO_2(H_2PO_4)_2$	503 (6.5)	524 (7.1)	548[a]	574[a]
U-malonate	494 (9)	515 (10)	539 (11)	565 (10)

a: fluorescence peaks not clearly resolved, estimated maximum.

is formed. After eight weeks the organo-phosphate ligands are completely replaced by dihydrogen phosphate which indicates that $UO_2(H_2PO_4)_2$ is more stable than the organo-phosphate complex.

X-ray absorption spectroscopy

EXAFS provides information about the number of neighboring atoms (N) and the distance to the neighboring atom (R) for a given near neighbor shell around the absorbing atom. As we will show below, it is capable of differentiating between organo-phosphate complexes and the dihydrogen-phosphate precipitate.

The U L_{III}-edge k^3-weighted EXAFS spectra and the corresponding Fourier transforms of the bacterial samples and the reference samples (U-AMP and $UO_2(H_2PO_4)_2$) and the theoretical fits are shown in Fig. 3. The data were fit using five scattering paths, including the U−O_{ax} multiple scattering path. The U−O_{ax} multiple scattering path was completely constrained to the U−O_{ax} path to minimize correlations in its fit parameters with those from the U−P path. In particular, the U−O_{ms} Debye–Waller factor was set equal to that for the U−O_{ax} path. Also, it was necessary to fix the Debye–Waller factor (bond length distribution width) for the U−P path. We chose to set it 0.05 Å as a minimum possible value after fitting to the entire data set with various constraints. The effect of all of these constraints is to minimize the measured amplitude of the U−P peak. The maximum number of the U−P pairs is more difficult to determine, but is likely within a factor of two of the minimum value based on a rough maximum of ∼ 0.10 Å for the U−P width. The results of the data analysis for U−O_{ax}, U−O_{eq} and U−P are summarized in Table 2. The coordination number of the phosphorus is estimated from the minimum and the maximum values within the error bars given in Table 2.

The U(VI) of the bacterial complexes with vegetative cells, heat killed cells, and spores of *B. sphaericus* is coordinated with two axial oxygen atoms at a distance of 1.768 to 1.776 Å (see Table 2) and 4.5 to 5.0 equatorial oxygen atoms at a distance of 2.37 Å (spores), 2.39 Å (vegetative cells), and 2.40 Å (heat killed cells). The uranyl ion can be coordinated by six, five or four equatorial oxygen atoms. The average bond lengths in hexagonal, pentagonal or square bipyramidal coordination polyhedra of inorganic U(VI) compounds are U−$O_{eq,\,hexagonal}$ = 2.46 (12) Å, U−$O_{eq,\,pentagonal}$ = 2.34 (10) Å, and U−$O_{eq,\,square}$ = 2.26 (8) Å[14]. The equatorial oxygen distances of the bacterial complexes agree with the average distance of the pentagonal coordination within the error bars. Furthermore, the spectra exhibit a peak about 3 Å (not phase corrected). Although this peak has a U−O_{ms} contribution, it is primarily caused by the scattering from the phosphorus atoms at a distance of 3.60–3.63 Å. The determination of the number of phosphorus atoms is complicated because the U−P contribution is very small and interferes strongly with the U−O_{ms} peak. Considering the maximum and the minimum value of the fitted amplitudes of the U−P scattering, we determined the number of phosphorus atoms to be about 1.0 ± 0.5. The X-ray absorption results of the bacterial U(VI) complexes are in good agreement with our recent results of the Pu(VI) complex with vegetative cells of *B. sphaericus* [5].

The U-AMP complex is coordinated with 6.0 ± 0.8 equatorial oxygen atoms at a shorter distance of 2.32 Å compared

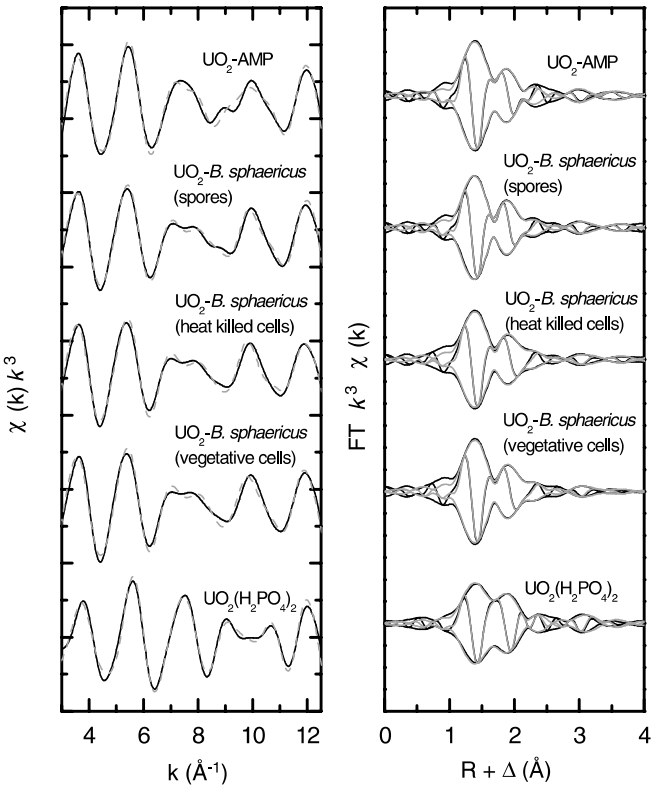

Fig. 3. U L_{III}-edge k^3-weighted EXAFS spectra (left) and the corresponding Fourier transforms (right) of the U(VI) bacterial complexes and the reference samples (U-AMP and $UO_2(H_2PO_4)_2$) and the theoretical fits (gray line). The outer envelope of the Fourier transforms is the amplitude and the oscillation inner line is the real part of the complex transform. The transform range is 2.5–13 $Å^{-1}$ and Gaussian narrowed with a 0.3 $Å^{-1}$ window. The fit range is 1.2–3.8 Å. The Fourier transforms are not corrected for EXAFS phase shifts, so that FT peak position of a given peak does not equal the pair distance *R*.

Table 2. EXAFS structural parameters of the bacterial UO_2^{2+}-complexes, the UO_2^{2+}-AMP complex, and $UO_2(H_2PO_4)_2$ (error in parentheses).

	U−O_{ax}			U−O_{eq}			U−P			E_0
	σ	R	A	σ	R	A	σ	R	A	
U-vegetative cells	0.040 (2)	1.775 (4)	2	0.11 (1)	2.39 (1)	5.0 (5)	0.05	3.63 (3)	1.0 (5)	−20 (1)
U-heat killed cells	0.046 (2)	1.776 (2)	2	0.11 (1)	2.398 (7)	4.8 (8)	0.05	3.63 (4)	1.0 (5)	−20 (1)
U-spores	0.039 (2)	1.768 (3)	2	0.10 (1)	2.371 (7)	4.5 (5)	0.05	3.60 (3)	1.0 (5)	−18 (1)
U-AMP	0.037 (2)	1.763 (2)	2	0.11 (1)	2.315 (7)	6.0 (8)	0.05	3.58 (2)	1.0 (5)	−15 (1)
$UO_2(H_2PO_4)_2$	0.060 (3)	1.778 (5)	2	0.05 (1)	2.285 (7)	3.1 (2)	0.05	3.60 (2)	2 (1)	−20 (1)

to the bacterial complexes. The U−P distance is 3.60 Å and the number of phosphorus atoms is also 1 ± 0.5. This might explain the almost identical fluorescence spectra of the U-AMP complex and the bacterial complexes. The complexation with the phosphate group causes a strong shift of the fluorescence bands and determines the fluorescence spectra. Differences in the coordination number and the distance of the equatorial oxygen atoms seem to have a minor influence on the fluorescence spectra.

In agreement to the laser fluorescence spectroscopy results, the EXAFS spectrum of $UO_2(H_2PO_4)_2$ differs significantly from those of the U-bacteria complexes and the U-AMP complex (see Fig. 3). The short distance of the equatorial oxygen atoms of 2.285 Å matches the average distance of a square bipyramidal coordination. The coordination number of the equatorial oxygen atoms is found to be 3.1 ± 0.2 which is lower than the expected coordination number of 4 determined by EXAFS measurement of $PuO_2(H_2PO_4)_2$ [5]. Furthermore, the amplitude of the U−P scattering is twice as high as those of the U-bacterial and the U-AMP complex. These significant differences in the structure of this model compound are also displayed by significant differences in the fluorescence spectrum.

Conclusion

The present results have proven that the interaction of U(VI) with bacterial cells of *B. sphaericus* leads to a formation of inner sphere complexes with organo-phosphate groups on the cell surface. Both methods, TRLFS and EXAFS, can clearly differentiate between organo-phosphate complexes and $UO_2(H_2PO_4)_2$. Our spectroscopic measurements have shown that by using fresh cells of *Bacillus sphaericus*, a possible formation of $UO_2(H_2PO_4)_2$ by complexation with $H_2PO_4^-$ excreted by the bacterial cells can be excluded. We observed no significant difference in the sorption behavior of vegetative and heat killed (but intact) cells and also in the coordination of the U(VI) complexes formed with the biomass. These results confirm that surface complexes are formed without metabolic activity involved. Though the small circular spores of *B. sphaericus* show a higher sorption related to their dry weight compared to vegetative and heat killed cells, the fluorescence and EXAFS spectra of the uranyl complexes are almost identical. We observed no significant difference in the coordination number and the distance of the oxygen and phosphorus atoms in the inner coordination sphere.

With increasing age of the bacterial sample, the cells starts lysing. During this process phosphate is excreted. The characterization of the released phosphate species by Raman spectroscopy [7] has shown that the feature of the Raman spectrum of the excreted phosphate is comparable to that of a KH_2PO_4 reference solution. The Raman shifts are significantly different from those of K_2HPO_4 and K_3PO_4. We conclude that the bacteria release $H_2PO_4^-$ during decomposition which leads to the formation of $UO_2(H_2PO_4)_2$. This is confirmed by time-resolved laser fluorescence spectroscopy. The fluorescence spectrum of U(VI) in the suspension with decomposed bacteria is consistent with the spectrum of the $UO_2(H_2PO_4)_2$.

The present results describe different kinds of interaction with *Bacillus sphaericus* as a function of age and status of the bacterial cells. These investigations are significant for a better description of natural systems, where living cells, spores, dead and also decomposed cells are present. Detailed information on the main interaction with different kinds of cells of *B. sphaericus* and their decomposition products will enable a better prediction on a possible impact of bacteria on the transport of actinides and can be a basis for developing new remediation strategies using bacteria for immobilization of actinides in waste deposits.

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